10/517684 IB/03/2641

AUSTRALIA

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Patent Office
Canberra

REC'D 18 AUG 2003
WIPO PCT

I, JONNE YABSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2002951020 for a patent by AUCKLAND UNISERVICES LIMITED as filed on 23 August 2002.

COMMONWEALTH OF AUSTRALIA
AUSTRALIA
PATENT OFFICE

WITNESS my hand this
Twenty-seventh day of February 2003

J R Yabsley

JONNE YABSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES

MEASUREMENT OF MELANOCORTIN PEPTIDES AND USES THEREOF

TECHNICAL FIELD

The present invention relates to methods that utilise melanocortin peptides, their receptors and biological response systems for the risk assessment and diagnosis of disease. The biological response systems are also utilised to screen for compounds that act as agonists or antagonists of melanocortin receptors.

BACKGROUND

Obesity and type 2 diabetes are major health problems worldwide and are a major threat to health and well-being. Over the last few years significant advances have been made with respect to the molecular determinants of energy balance and insulin resistance. Critical elements of this control system are hormones secreted in proportion to body fat, including leptin and insulin, and their central nervous system targets such as neuropeptide Y and the hypothalamic melanocortin system. Recently proopiomelanocortin and MC4-R have been identified as key targets mediating leptin's activities in the hypothalamus.

Pro-opiomelanocortin (POMC), produced in the pituitary and brain and to a lesser extent in numerous peripheral tissues including skin, pancreas and testis, is the large precursor protein from which melanocortin peptides α-melanocyte stimulating hormone (MSH) and adrenocorticotropin (ACTH) and fragments thereof, are derived. The products of POMC undergo a series of complex, tissue specific processing events such as further proteolytic cleavages, phosphorylation, α-amidation and NH_2-terminal acetylation which influence their biological activities. $ACTH_{1-13}NH_2$ exists as α-MSH and desacetyl-α-MSH. α-MSH, which is acetylated at the N-terminus and amidated at the COOH terminus, is a post translationally modified derivative of $ACTH_{1-13}NH_2$ (desacetyl-α-MSH). The acetylation reaction to form α-MSH is associated with the secretory process; its highest activity is present in the pituitary gland and certain brain regions.

The functional significance of N-terminal acetylation of $ACTH_{1-13}$ in the central nervous system is unknown although it may acutely regulate $ACTH_{1-13}$ action. N-terminal acetylation of desacetyl-α-MSH to form α-MSH enhances some activities of $ACTH_{1-13}$ and virtually eliminates others. α-MSH injected daily to rats is 10 -100 fold more effective than desacetyl-α-MSH at increasing pigmentation, arousal, memory, attention, and excessive grooming. Desacetyl-α-MSH, however, is more effective than α-MSH at blocking opiate analgesia and opiate receptor binding *in vivo*. α-MSH and desacetyl-α-MSH also differentially affect feeding and weight gain. Weight gain of *agouti* obese mice is increased by subcutaneously administered desacetyl-α-MSH, as is food intake and fat pad weight, but α-MSH injections do not significantly increase food intake or body weight.

Although melanocortin peptides and melanocortin receptors have been identified as playing pivotal roles in regulating food intake, metabolism and energy homeostasis, these advances in our understanding of energy homeostasis have not yielded clinically applicable parameters with which to predict or diagnose pathological imbalances that lead to obesity and/or type 2 diabetes. There is a need therefore for methods which would assist in the analysis and monitoring of energy metabolism, feeding and weight gain patterns.

It is an object of the present invention to ameliorate at least some of the disadvantages of the prior art methods, or at least provide useful alternatives.

SUMMARY OF THE INVENTION

Just as the measurement of "good" (HDL) and "bad" (LDL) cholesterol predicts cardiovascular risk, we have discovered that the balance/abundance/status of melanocortin peptides is predictive and/or diagnostic of imbalances in energy homeostasis, disturbances in feeding/weight gain patterns, obesity and/or type II diabetes. For example, the abundance and/or ratio of the melanocortin peptides α-MSH and

desacetyl-α-MSH predicts feeding/weight gain patterns, imbalances in energy homeostasis and obesity risk. This novel approach involves the use of a biological response system that processes stimulus through melanocortin receptors, and which outputs information through various response parameters.

For the purposes of the invention herein described, the term "biological response system" includes any whole animal, organ, tissue or cell which is able to respond to a melanocortin peptide or an effector molecule generated by a response to a melanocortin peptide.

For the purposes of the invention herein described, the term "response parameter" includes a cellular product (which may be a protein, nucleic acid, lipid, carbohydrate or a combination of these), or a measurable cellular event, resulting from interaction of the biological response system with a melanocortin peptide. Mass spectrometry or currently commercially available gene expression arrays may be used to monitor these response parameters, among other techniques.

Although we do not wish to be bound by any particular theory, when the biological response system is treated with melanocortin peptides, or a sample containing melanocortin peptides, the profile or "fingerprint" of response parameters resulting from melanocortin receptor stimulus may reflect the melanocortin peptide balance/abundance/status of the sample. A comparison of the fingerprints of response parameters resulting from normal subjects and obese individuals and/or type II diabetics, or individuals with an imbalance in energy homeostasis and/or disturbance in feeding/weight gain patterns provides additional information, by way of profile databases, that may be used to predict imbalance in energy homeostasis and/or disturbance in feeding/weight gain patterns or the risk of onset of obesity and/or type II diabetes or that may be diagnostic of these conditions.

For the purpose of the invention described herein, the term "profile" or "fingerprint of response parameters" is a reference to one or a plurality of response parameters that may be ascertained by various techniques, which

are indicative of an imbalance in energy homeostasis and/or disturbance in feeding/weight gain patterns or the risk of onset of obesity and/or type II diabetes or obesity and/or type II diabetes.

The response parameters that are profiled in the biological response systems may be the result of a primary response by the system to stimulus by melanocortin peptides, or, they may be the result of a secondary response following the primary response to melanocortin peptides. The response profile may be utilised to monitor treatments used for obesity and/or type II diabetes. One such treatment may involve the administration of melanocortin peptides, and/or agents that influence their abundance/balance. The profiles may also be used to monitor the onset obesity and/or type II diabetes, the efficacy of treatment, relapse or progression of obesity and/or type II diabetes or imbalance in energy homeostasis and/or disturbance in feeding/weight gain patterns. The profile of parameters may therefore be adopted as a clinician's tool to assess risk of developing disease, diagnose disease, monitor disease and monitor treatment of disease.

The biological response system is also useful to screen for compounds that are effective in the treatment of imbalances in energy homeostasis and/or disturbances in feeding/weight gain patterns or obesity and/or type II diabetes. The system would also be useful to screen for compounds that act as agonists or antagonists of melanocortin receptors. The response to test compounds, reflected in the resulting profile of response parameters, may be monitored by mass spectrometry or currently commercially available gene expression arrays, among other techniques. Such compounds are potential candidates for the treatment or prevention of obesity and/or type II diabetes, or an imbalance in energy homeostasis, or a disturbance in feeding/weight gain patterns, or other metabolic imbalances brought about by disturbances in melanocortin peptide balance/abundance/status and the resultant receptor response.

According to a first aspect of the invention, there is provided a method of determining the melanocortin peptide status of a sample

comprising contacting the sample with a biological response system wherein the resulting profile of response parameters produced by the biological response system indicates the melanocortin peptide status of the sample.

According to a second aspect of the invention, there is provided a method of assessing risk of developing obesity and/or type 2 diabetes in a mammal comprising contacting a sample obtained from a mammal with a biological response system wherein the resulting profile of response parameters is indicative of the risk of developing obesity and/or type 2 diabetes.

According to a third aspect, the invention provides a method of diagnosing obesity in a mammal comprising contacting a sample obtained from a mammal with a biological response system wherein the resulting profile of response parameters is diagnostic of obesity.

According to a fourth aspect, the invention provides a method of assessing the risk of developing an imbalance in energy homeostasis or a disturbance in feeding/weight gain patterns in a mammal comprising contacting a sample obtained from a mammal with a biological response system wherein the resulting profile of response parameters is indicative of the risk of developing an imbalance in energy homeostasis or a disturbance in feeding/weight gain patterns.

According to a fifth aspect, the invention provides a method of diagnosing an imbalance in energy homeostasis or a disturbance in feeding/weight gain patterns in a mammal comprising contacting a sample obtained from the mammal with a biological response system wherein the resulting profile of response parameters is diagnostic of an imbalance in energy homeostasis or a disturbance in feeding/weight gain patterns.

According to a sixth aspect, the invention provides a method of monitoring treatments for obesity and/or type II diabetes in a mammal comprising contacting a sample obtained from a mammal treated with such treatments with a biological response system wherein the resulting profile of

response parameters is indicative of the effect of such treatments on obesity and/or type II diabetes.

According to a seventh aspect, the invention provides a method of monitoring treatments for imbalances in energy homeostasis and/or disturbances in feeding/weight gain patterns in a mammal comprising contacting a sample obtained from a mammal treated with such treatments with a biological response system wherein the resulting profile of response parameters is indicative of the effect of such treatments on imbalances in energy homeostasis and/or disturbances in feeding/weight gain patterns. Such treatments may involve the administration to a mammal, melanocortin peptides and/or agents that influence their abundance/balance. It is understood that any treatment used for obesity and/or type II diabetes may impact on the melanocortin peptide status and may be measured according to the invention described herein.

According to an eighth aspect, the invention provides a method of assessing the risk of developing obesity and/or type II diabetes comprising analysing the profile of response parameters in a sample from a test subject by comparing it with

(i) the profile of a sample from a normal subject and

(ii) the profile of a sample from an obese and/or type II diabetic subject,

wherein resemblance of the profile of the sample obtained from the test subject to that of the profile in (ii) above, is indicative of that subject being at risk of developing obesity and/or type II diabetes.

According to a ninth aspect, the invention provides a method of assessing the risk of developing and/or having an imbalance in energy homeostasis and/or disturbance in feeding/weight gain patterns comprising analysing the profile of response parameters in a sample from a test subject by comparing it with

(i) the profile of a sample from a normal subject and

(ii) the profile of a sample from a subject with an imbalance in energy homeostasis and/or disturbances in feeding/weight gain patterns.

wherein resemblance of the profile of the sample obtained from the test subject to that of the profile in (ii) above, is indicative of that subject at risk of or having an imbalance in energy homeostasis and/or disturbances in feeding/weight gain patterns.

According to a tenth aspect, the invention provides a method of screening for compounds that act as agonists or antagonists of melanocortin receptors comprising treating a biological response system with test compounds and measuring the resulting profile of response parameters that are indicative of agonist or antagonist activity to melanocortin receptors.

According to an eleventh aspect, the invention provides a method of screening for compounds that may be useful in the treatment of obesity and/or type II diabetes comprising treating a biological response system with test compounds and measuring the resulting profile of response parameters that are indicative of the desired response for the treatment of obesity and/or type II diabetes.

According to an eleventh aspect, the invention provides a method of screening for compounds that may be useful in the treatment of an imbalance in energy homeostasis or a disturbance in feeding/weight gain patterns comprising treating a biological response system with test compounds and measuring the resulting profile of response parameters that are indicative of the desired response for the treatment of an imbalance in energy homeostasis or a disturbance in feeding/weight gain patterns.

Preferably, the biological response system is an *in vitro* cell or organ culture capable of responding to melanocortin peptides. The preferred *in vitro* cell cultures are cultures of primary rat osteoblasts, or the UMR106.06 rat osteosarcoma cell line, or the GT1-7 mouse hypothalamic cell line. Any cell line or primary culture of cells that expresses melanocortin receptors, or any combination of such cell lines, may also be used as an *in vitro* biological response system. Some of these cell lines are 3T3-L1 adipocytes, melanocytes, L6 myocytes, B16 melanoma cells, and anterior pituitary cell cultures. Any cell line or primary culture of cells that express

melanocortin receptors, or any combination of such cell lines, that are capable of producing a differential response that distinguishes obese individuals and/or type II diabetics, or individuals at risk of developing obesity and/or type II diabetes, or individuals suffering from an imbalance in energy homeostasis or disturbance in feeding/weight gain patterns, from normal individuals may be used as an *in vitro* biological response system. As the given list is not exhaustive of cell lines or primary cell cultures that express melanocortin receptors, the *in vitro* biological response system described herein is not limited to the use of these. The biological response system may also be an *in vivo* system. Examples of *in vivo* systems include the hypothalamus of a mammal and/or other tissue(s) that are capable of responding to melanocortin peptides.

Of course, it will be understood that a whole animal may be used as an *in* vivo biological response system. In the case where a whole animal is used as an *in vivo* biological response system the response parameters may be feeding frequency and/or body weight gain. Further, samples may be introduced in to the animal biological response system, and tissues and/or organ samples may be obtained from the animal biological response system, which samples may be analysed for the relevant response parameters.

The preferred response profile or fingerprint is one or more proteins or cellular events which differentiate between normal individuals and those at risk of developing obesity and/or type II diabetes, or those suffering from obesity and/or type II diabetes, or those with an imbalance in energy homeostasis, or disturbance in feeding/weight gain patterns.

The preferred response parameters are proteins expressed by the biological response system. Proteins expressed by the biological response system includes but are not limited to stress proteins such as heat shock protein homologue, enzymes such as glyceraldehyde-3-phosphate-dehydrogenase, aldo-keto reductase, citrate synthase, creatine kinase, pyruvate synthase alpha-chain, f1 ATPase beta-chain, and cytoskeletal proteins such as tubulin beta-chain. Other proteins which may be used as

response parameters include but are not limited to proteins involved in the melanocortin peptidergic axis, proteins involved in signalling pathways, enzymes, and membrane-bound proteins. Extracellular effector molecules may also be suitable response parameters.

BRIEF DESCRIPTION OF THE FIGURES.

Figure 1. ***Alpha-MSH but not desacetyl-α-MSH administered i.c.v. significantly decreased food intake.*** Food intake was measured over 3h following lateral ventricle injections of vehicle (PBS), 10μg α-MSH, or 10μg desacetyl-α-MSH to food deprived Wistar rats. (PBS, n = 9; α-MSH, n = 7; desacetyl-α-MSH, n = 10). Alpha-MSH significantly decreased food intake to 70% of PBS treated control (*, significantly different from PBS, $p < 0.05$, one way ANOVA). Desacetyl-α-MSH has no significant effect on feeding, but there was a trend for a reduction in food intake.

Figure 2. ***A higher dose of desacetyl-α-MSH compared to α-MSH administered i.c.v. significantly decreased food intake.*** Food intake was measured over 3h following lateral ventricle injections of vehicle (PBS), 10μg α-MSH, or 50μg desacetyl-α-MSH to food deprived Wistar rats. (PBS, n = 11; α-MSH, n = 11; desacetyl-α-MSH, n=11). (*, significantly different from PBS< $p < 0.05$, one way ANOVA).

Figure 3. ***Desacetyl-α-MSH significantly slowed body weight change in neonatal rats.*** Neonatal rats were injected subcutaneously with PBS (n=36), α-MSH (n=27) or desacetyl-α-MSH (n=27) (0.3 μg/g body weight/day) for their first 14 days of life. There were no significant differences in body weight over 14 days between PBS and α-MSH treated pups.

Neonatal rats treated with desacetyl-α-MSH for 14 days grew significantly slower than either PBS or α-MSH treated pups ($p < 0.05$, GLM repeated measures analysis of variance, SAS system).

Figure 4 ***RT-PCR shows MC2-R, MC4-R and MC5-R expression in primary rat osteoblast cells.*** Lane 2, MC2-R PCR product (290p); lane 4, MC4-R PCR product (554bp); lane 6, MC5-R PCR product (290bp); controls of specificity were the absence of RT in the reverse transcription reaction mixture (lane 3, MC2-R; lane 5, MC4-R; lane 7, MC5-R). The primers used are shown in Table 1. The PCR products were run on a 2% agarose gel alongside a HindII-EcoRI digested lambda DNA molecular weight marker (lane 1).

Figure 5 ***Northern blot analysis showed MC4-R mRNA transcripts in primary rat osteoblasts.***
Poly (A^+) mRNA (5 μg) from rat brain (lane 1) and primary rat osteoblasts (lane 2) were separated by formaldehyde-agarose gel electrophoresis (1.2%), transferred to a nylon membrane and probed with a ^{32}P labeled specific rat MC4-R DNA fragment. A digital image was obtained with a Storm imaging system screen and scanner. An RNA ladder was run on the gel and used to determine the mRNA sizes (2.0 – 2.6).

Figure 6 ***Ribonuclease Protection Assay shows MC4-R mRNA expression in UMR106.06 and primary rat osteoblast cells.*** Lane 2, full length rat MC4-R riboprobe (562bp), probe incubated with: lane 3, 1 μg/ml RNase A and 50 U Rnase T1; lane 4, 10 μg tRNA; lane 5, 10 μg rat brain poly (A^+) mRNA, lane 6, 10 μg primary rat osteoblast poly (A^+) mRNA; lane 7, 10 μg UMR106.06 poly (A^+) mRNA. The labeled fragments

were run on a 6% polyacrylamide gel alongside a radiolabeled 123bp DNA Ladder (GIBCO BRL) (lane 1). The data shown are representative of three independent experiments.

Figure 7 ***Alpha-MSH stimulation of rat primary osteoblast proliferation.*** . Growth arrested primary rat osteoblasts were stimulated with increasing doses of α-MSH and [^{3}H] thymidine uptake (a) and changes in cell number (b) measured. Data are expressed as mean ± SEM. Significant difference from control; * = $p<0.04$, ** $p< 0.001$

Figure 8. ***Desacetyl-α-MSH and ACTH1-24 antagonise α-MSH stimulated stimulation of thymidine incorporation into cultures of rat primary osteoblasts.*** Growth arrested primary rat osteoblasts were stimulated with either 10^{-7}M or 10^{-8}M α-MSH alone (a,b), 10^{-7}M desacetyl-α-MSH alone (a), $ACTH_{1-24}$ alone (b), or combinations of α-MSH and desacetyl-α-MSH (a) or α-MSH and $ACTH_{1-24}$ (b) and [^{3}H] thymidine uptake measured. Data are expressed as mean ± SEM. Significant difference from control; * = $p<0.04$, ** $p< 0.001$

Figure 9. ***Biphasic Dose response curve for treatment of UMR106.06 with alpha-MSH.*** UMR106.06 rat osteosarcoma cells were stimulated with 10^{-6} to 10^{-12} alpha-MSH and the [^{3}H] thymidine uptake measured.

Figure 10. ***Dose response curve for treatment of UMR106.06 with desacetyl-alpha-MSH.*** UMR106.06 rat osteosarcoma cells were stimulated with 10^{-6} to 10^{-12} desacetyl-alpha-MSH and the [^{3}H] thymidine uptake measured.

Figure 11A to C. ***Proteome analysis***. Figures 11 A to C show differences in protein profiles after treament with alpha-MSH and desacetyl-alpha-MSH.

Figure 12 ***Effects of alpha-MSH on Thymidine incorporation in Chondrocyte monolayers***. The figure shows increased thymidine incorporation (interpreted as increased cell proliferation) in response to stimulation by alpha-MSH.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention is based on a surprising observation that the balance/abundance/status of MSH peptides in the circulation, may correlate with, and be predictive of, the development of an imbalance in energy homeostasis, disturbance in feeding/weight gain patterns and ultimately obesity and diabetes. The absolute levels of individual MSH peptides may also serve this purpose. The absolute levels of combined MSH peptides may also serve this purpose.

Preferred embodiments of the invention will now be described by way of example only with reference to the following examples.

EXAMPLES

Example 1: *In vivo* biological response of the hypothalamus to alpha-MSH and desacetyl-alpha-MSH peptides.

Alpha-MSH and desacetyl-α-MSH both couple melanocortin receptors to either adenylyl cyclase or calcium-signalling pathways *in vitro*. To characterise the signal transduction pathways engaged by α-MSH and desacetyl-α-MSH *in vivo*, rats received an intracerebroventricular (i.c.v.) injection of either phosphate buffered saline (PBS), α-MSH or desacetyl-α-

MSH. Three hours later, food intake was measured and hypothalamic tissues were collected for 2D gel electrophoresis-based proteome analysis.

Intracerebroventricular injection of melanocortin peptides in adult rats.

Animals:

Adult male Wistar rats (50-60 days old, 230-260g at the beginning of the experiment) were maintained in individual cages under controlled temperature (23°C) and reverse lighting (1000-2200 lights off). Standard laboratory chow (NZ Stockfeed Ltd) and tap water were available *ad libitum* during the adaptation phase. During this time animals were handled daily to minimize the effects of stress on food intake during experiments. Body weight was measured daily before, and one week after cannulation. Any animal showing signs of illness, such as weight loss, poor grooming, or decreased activity, was removed from the study. All animal procedures undertaken were approved by the Animal Ethics Committee of the University of Auckland.

Cannula placement:

After 7 days of adaptation, animals were subject to cannula placement surgery under 3% halothane /O_2 anaesthesia. A permanent lateral ventricle infusion cannula (6-mm 21 gauge) was placed on top of the dura at 7.5 mm anterior from stereotaxic zero, 1.5 mm to the right of the mid-sagittal line, and secured to the skull with dental cement. Animals were allowed at least 7 days to recover from surgery before injections.

ICV infusion of melanocortin peptides.

Rats were fasted overnight before the day of experiment. Starvation serves to increase baseline food intake during the initial few hours of testing

melanocortin peptide effects on food intake, thereby providing a greater range in which the effect of the anorectic agent α-MSH could be demonstrated.

Under 3% halothane /O_2 anaethesia rats were infused icv through a 12-mm 27-gauge needle, connected to 20-cm length tubing attached to a syringe. Infusions were performed in the early dark phase between 1000 and 1130 hr using motor driven infusion pumps at a rate of 1.0 µl/min over 10 min. Movement of a 0.2 ml air-space introduced between the 0.9% saline solution filling the PE10 tubing-syringe system and the test solution served as an indicator of a successful infusion. At the end of each experiment animals were euthanised by pentobarbital overdose, and cannula placement was confirmed by visual inspection of the cannula tip location within the brain ventricular system.

Proteome analysis

Proteome analysis showed that the expression of 14 proteins were significantly different between PBS and α-MSH, and 20 proteins were significantly different between PBS and desacetyl-α-MSH treated groups ($p<0.05$, non-parametric/Mann-Whitney U test). Only one of these proteins was common to α-MSH and desacetyl-α-MSH. A combination of Reverse-phase HPLC followed by Edman protein sequencing, and peptide mass fingerprinting technique using MALDI-TOF mass spectrometry were used to identify the proteins of interest. The proteomic data provide a snap-shot of the protein expression patterns in the hypothalamus 3 hours post i.c.v. administration of the melanocortin peptides. The expression of different hypothalamic proteins following administration of either α-MSH or desacetyl-α-MSH supports the hypothesis that these peptides activate different biological responses *in vivo* by activating different molecular and cellular signalling pathways. (Figures 11 A to 11C)

Measurement of food intake:

Following infusion, the cannula was left in place for 1 min, removed, and the animal returned to its cage with fresh pre-weighed food and water. At 3 h post-injection, the pellets and collected food spillage in the cage, were weighed and this weight was subtracted from the initial weight to quantify the amount of food eaten over 3 h.

***Statistical analysis*:**

The significance of treatment effects was evaluated using one-way ANOVA (Systat10 package)

RESULTS

Alpha-MSH is more potent than desacetyl-α-MSH at inhibiting food intake.

Alpha-MSH (10μg) administered i.c.v to food deprived adult rats just prior to the 12h dark cycle significantly reduced food intake over 3h compared to PBS treated control animals (α-MSH, n = 7; PBS, n= 9; $p<0.05$). There was a trend for desacetyl-α-MSH (10μg) to also decrease food intake (n=10) over 3h, but this was not significantly different from the PBS treated control group of rats.

A 5-fold higher dose of desacetyl-α-MSH (50μg) did significantly reduce food intake over 3h compared to PBS treated control animals (desacetyl-α-MSH, n = 11; PBS, n= 11 $p<0.05$) in a second independent study. In this study α-MSH (10μg) again significantly inhibited food intake over 3h compared to PBS treated control animals (α-MSH, n = 11; $p<0.05$) .

Pups treated with desacetyl-α-MSH (n=27) grew significantly slower than either vehicle control (n=36) or alpha-MSH treated pups (n=27) (p,0.05, repeated measures analysis of variance, SAS).

Example 2: *In vivo* biological response to the subcutaneous administration of alpha-MSH and desacetyl-alpha-MSH peptides in rats.

The activity of alpha-MSH and desacetyl-alpha-MSH when administered peripherally was measured by subcutaneous administration to postnatal rats for 14 days.

Animals:

Adult female Wistar rats were housed in plastic cages and kept on a 12-h dark/light cycle. Animals received tap water and rat pellets *ad libitum* and were mated with males of the same strain. Each litter of new-born Wistar rats was culled to 9 pups per mother.

Subcutaneous injections of melanocortin peptides:

Each litter was assigned to a treatment group; vehicle, phosphate buffered saline (PBS), α-MSH (0.3μg/g body weight/day), or desacetyl-α-MSH (0.3□g/g body weight/day). PBS or freshly prepared peptide solutions made up freshly in PBS containing 0.1% BSA were injected subcutaneously once per day in a volume of 40 μl for 14 days. Animals were injected on day 14 and 1h later they were euthanised using sodium pentobarbital.

Measurement of body and organ weights:

Rats were weighed at birth and then every 2 days prior to injection of peptides. Body weights were recorded on day 14 before injection and again when they were euthanised. The following organs were dissected and weighed: brain, heart, kidney, liver, lung, spleen.

Statistical analysis:

We tested for liner relationships between organ weights and body weights using regression analysis of the organ weights measured against final body weight on day 14. There were significant linear relationships between organ weights and body weights for the following tissues: brain,

spleen, heart, kidney and liver. There was no significant regression between lung weight and body weight. For those organs where their weight was linearly correlated to body weight, treatment effects on organ weight changes were analysed using ANCOVA with body weight as the co-variate.

Differences in body weight were analysed using a General Linear Model with repeated measures . Significance was assumed at the $P < 0.05$ level.

Desacetyl-α-MSH significantly slowed body weight change in neonatal rats.

Three litters of neonatal rats injected daily with desacetyl-α-MSH (0.3 μg/g body weight/day) for their first two weeks of life grew significantly slower than control pups injected daily with PBS (4 litters). In contrast, α-MSH (0.3 μg/g body weight/day) injected daily in neonatal rats (3 litters) had no significant effect compared to control pups injected with PBS. Body weight data obtained from these subcutaneous injections of melanocortin peptides were analysed as a nested within nested design, with the following independent factors: Treatment effects, Litter (Treatment) effects, and Rat (Litter * Treatment) effects. This analysis allowed the separation of sources of variation due to treatment effects, from between litter and between individual rat, differences. Data were analysed using a General Linear Model with repeated measures. Both α-MSH and desacetyl-α-MSH treated neonatal rats appeared to catch up on body weight from day 12 compared to control PBS treated rats.

Different effects of subcutaneously administered α-MSH and desacetyl-α-MSH on organ weights in neonatal rats.

Both α-MSH and desacetyl-α-MSH (0.3 μg/g body weight/day) administered subcutaneously daily for 14 days to neonatal rats, significantly decreased brain weight compared with control PBS treated animals. Alpha-MSH significantly decreased kidney weight but desacetyl-α-MSH had no significant effect on kidney weight. Desacetyl-α-MSH, however,

significantly increased spleen weight but α-MSH had no significant effect on spleen weight.

Example 3: *In vitro* melanocortin receptor-mediated biological response system: *In vitro* biological response of primary rat osteoblasts and UMR106.06 rat osteosarcoma cells to melanocortin peptides.

Materials:

The melanocortin peptides, $ACTH_{1-24}$, desacetyl-α-MSH and α-MSH were purchased from Bachem California (CA, USA). The production of recombinant mouse agouti protein has previously been described (Willard, 1995 #760). [^{3}H] Methyl thymidine was purchased from Amersham Life Science (Buckinghamshire, U.K.).

Cells:

Rat osteosarcoma UMR106.06 cells were grown in Dulbecco's modified Eagle's Medium (DMEM) (GIBCO BRL, Rockville, MD) supplemented with 10% fetal calf serum (FCS) (In Vitrogen, Auckland, NZ) and 50 U/ml penicillin plus 50 μg/ml streptomycin. Cells were maintained at 37°C in 5% CO_2 and passaged every week.

Primary rat osteoblasts were isolated from 20 day fetal rat calvariae. (The use of animals for these studies was approved by the Auckland Animal Ethics Committee.) Calvariae were excised and the frontal and parietal bones, free of suture and periosteal tissue, were collected and sequentially digested using collagenase as previously described (Cornish, 1999 #1792). Primary rat osteoblasts were grown in DMEM supplemented with 10% FCS, 50 U/ml penicillin and 50 μg/ml streptomycin. After 48 hour, the medium was changed to MEM. Confluence was reached within 5-6 days, at which time the cells were subcultured into 10cm culture plates for RNA preparation or 24 well plates for proliferation assays.

Preparation of mRNA

Total RNA was extracted from adult rat brain, skin, UMR106.06, or primary rat osteoblast cells using the guanidinium thiocyanate method (Chirgwin, 1979 #129). Poly $(A)^+$ mRNA was purified from the total RNA using the PolyATract mRNA Isolation System (Promega, Madison, WI).

Northern Blot Analysis

Primary rat osteoblast poly $(A)^+$ (5µg) and rat brain poly $(A)^+$ were size separated alongside lamda EcoRI/HindIII markers by electrophoresis on a 2.2M formaldehyde-1.2% agarose gel, transferred to a Magnacharge Nylon membrane (MSI, Westborough, MA), and hybridised with a rat specific MC4-R gene DNA fragment spanning transmembrane domains III and VII (Mountjoy, 1994 #656). Hybridisation conditions were 50% formamide, 1mM NaCl, 50mM Tris-HCl (pH 7.5), sodium pyrophosphate (0.1%), SDS (0.2%), salmon sperm DNA (100µg/ml), 10x Denhardt's and 10% dextran sulfate at 42°C for 18h. A digital image of MC4-R transcripts was obtained after 10 days exposure with a phosphoscreen by using the Storm imaging system scanner (Molecular Dynamics).

PCR amplification of reverse transcribed mRNA (RT-PCR)

Poly $(A)^+$ mRNA was DNase treated twice using 10 U RQ1 RNase-free DNase (Promega Corp., Madison, WI) per mg poly $(A)^+$ mRNA for 30 min at 37°C each time. First strand cDNA was synthesised using 200 U SuperScript II RNaseH$^-$ reverse transcriptase (GIBCO BRL, Rockville, MD) and oligo $(dT)_{12-18}$ (Pharmacia Biotech AB, Uppsala, Sweden) at 42°C for 1h in a final volume of 20 µl. To test for DNA contamination of the RNA, a reaction was carried out with 1 µg poly $(A)^+$ mRNA and all the reagents but no reverse transcriptase (control reaction). The cDNA and control reaction (2 µl) were used as templates for PCR with rat melanocortin receptor specific oligonucleotides described in Table 1. The PCR conditions were 94°C for 3 min, 40 cycles of 94°C for 40 sec, annealing for 40 sec, and 72°C for 1 min, followed by 72°C for 10 min. The amplified cDNA products

were separated on a 1.2% agarose gel alongside a EcoRI-HindIII-digested lambda DNA ladder and stained with ethidium bromide.

Ribonuclease protection assay

The cDNA templates used to synthesise the antisense rMC4-R and rMC1-R riboprobes were generated from 562 and 270 bp respectively, nucleotide DNA fragment spanning transmembrane I to VII and III to VI domains subcloned into pBKS (Stratagene). These recombinant DNA templates were linearised with EcoRI and SalI transcribed with [α-^{32}P]UTP (Amersham Life Science (Buckinghamshire, U.K.) using T 7 RNA polymerase to generate ^{32}P-labeled cRNA probes. Rat brain or skin, UMR106.06, and primary rat osteoblast poly (A)$^+$ mRNA (10 µg) were treated with 2 U RNase-free DNaseI (Boehringer Mannheim, Indianapolid, IN) at 37°C for 50 min and the RNA was precipitated. The RNA pellet was resuspended in 20 µl hybridization buffer (80% formamide, 40 mM PIPES pH 6.4, 400 mM NaCl, 1mM EDTA) with 5 x 10^5 cpm of ^{32}P-labeled riboprobe, denatured at 85°C for 5 min and hybridized at 45°C overnight. The hybridised RNA was digested with 40 µg RNase A and 50U Rnase T1 at 37°C for 30 min. The protected RNA fragments were analyzed on a 6% denaturating polyacrylamide gel alongside a ^{32}P-labeled 123-bp DNA ladder (10^5 cpm). A digital image of ^{32}P-labeled fragments was obtained using a Storm imaging system.

In Situ Hybridisation

Neonatal mouse calvariae, tibial and femoral bone were collected from 1-2 and 6 day old Swiss mice that had been euthanised by cervical dislocation while under halothane anesthesia (approved by the Auckland Animal Ethics Committee). The bones were dissected free of adherent soft tissues and fixed in 4% paraformaldehyde for 24h at 4°C prior to decalcification (15% EDTA, 4% paraformaldehyde) for 72 h at 4°C. They were then transferred to10% sucrose, 4% paraformaldehyde overnight at 4°C before being embedded in OCT and stored frozen at –80°C. Five series of 20 µM of either cross sectional or longitudinal sections were cut on the cryostat and mounted onto polysine coated microscope slides (Biolab Scientific, NZ) and in situ hybridisation performed as previously described (Mountjoy, 1994 #656). Bone sections were hybridised with ^{33}P labelled

cRNA antisense rat MC4-R (628bp). Sections were hybridised in 65% formamide in 0.26M NaCl, 1.3x Denhardt's, 13mM Tris-HCl pH 8.0, 1.3mMEDTA, 13% dextran sulphate at 60-65°C for 18 hours. Sections were washed and coated with emulsion for autoradiography. Following the developing of these slides, the sections were stained with haematoxylin and eosin and then photographed under darkfield on a Leica Microscope (Leitz DMRBE). One series of sections from each case was not subjected to in situ hybridization but was counterstained with haematoxylin and eosin and used for the identification of structures and bone cell type.

Primary rat osteoblasts proliferation assays

Primary rat osteoblasts were subcultured into 24 well plates at a density of 5×10^4 cells/ml/well in MEM, 5% FCS for 24 hours. Cells were growth arrested in MEM, 0.1% bovine serum albumin (BSA) for 24 hour and then fresh media and experimental compounds were added for a further 24 hours. Cells were pulsed with [^{3}H]thymidine (0.5µCl/well) 2 hours before the end of the experimental incubation. The experiment was terminated and both cell numbers and thymidine incorporation were assessed. Cell numbers were analysed by detaching cells from the wells by exposure to trypsin/EDTA (.05%/0.53mM) for approximately 5 minutes at 37°C. Counting was performed in a hemocytometer chamber. Results are expressed per well. [^{3}H]Thymidine incorporation was analysed by washing the cells in MEM followed by the addition of 10% trichloroacetic acid. The precipitate was washed twice with ethnol:ether (3:1) and the wells desiccated at room temperature. The residue was dissolved in 2M KOH at 55°C for 30 minutes, neutralized with 1M HCl, and an aliquot counted for radioactivity. Results are expressed as dpm per well. Each experiment was performed at least three times using experimental groups consisting of at least six wells.

Statisitics

Data are presented as mean ± SEM. The significance of differences between groups was determined using Student's t tests for unpaired data and a 5% significance level. The comparisons to be made in each experiment were specified a priori, so no adjustment for multiple comparisons was necessary. Where several experiments have been shown in one figure, the data are expressed as the ratio of results in treatment groups to those in the control group

and the 'P' values shown were calculated using the data from the individual experiments, before the data were pooled.

RESULTS

MC4-R mRNA is expressed in UMR106.06 and primary rat osteoblast cells.

Four different methods confirmed expression of MC4-R mRNA in UMR106.06 and rat primary osteoblast cells. First, RT-PCR, using rat specific MC4-R oligonucleotides amplified the correct size DNA fragment from poly A^+ mRNA and not from genomic DNA. Second, Northern blot analysis of rat primary osteoblast poly (A^+) mRNA (5 µg) showed a broad band of MC4-R mRNA transcripts between 2.0 and 2.6 kb, the same size as seen in rat brain, albeit of much lower abundance than in brain. Third, RPA's confirmed MC4-R mRNA expression in UMR106.06 and primary rat osteoblast cells. Finally, we used *in situ* hybridisation to localise MC4-R mRNA expression in the periosteum of 1-2 and 6 day old Swiss mouse calvariae, tibia, and femoral bones.

MC2-R and MC5-R mRNA are expressed in UMR106.06 and rat primary osteoblast cells.

RT-PCR, using rat specific MC2-R and MC5-R oligonucleotides amplified correct size DNA fragments from 1 µg UMR106.06 and 1 µg primary rat osteoblast cell poly A^+ mRNA, but not from genomic DNA.

Alpha-MSH, but not desacetyl-α-MSH nor $ACTH_{1-24}$ stimulates proliferation of primary rat osteoblasts.

Alpha-MSH (10^{-9} – 10^{-7} M) significantly increased thymidine incorporation into growth arrested primary rat osteoblasts. Over a similar range of concentrations alpha-MSH also increased osteoblasts cell numbers. Desacetyl-α-MSH (10^{-7} M) and $ACTH_{1-24}$ (10^{-7} M) did not stimulate thymidine incorporation or cells numbers in growth arrested rat primary osteoblasts.

Desacetyl-α-MSH and $ACTH_{1-24}$ antagonise α-MSH stimulated proliferation of primary rat osteoblasts.

Desacetyl-α-MSH (10^{-7}M) inhibited two doses of α-MSH (10^{-8}M and 10^{-7}M) from stimulating [^{3}H] thymidine uptake into growth arrested rat primary osteoblasts (Figure 6a). $ACTH_{1-24}$ (10^{-7} M) inhibited two doses of α-MSH (10^{-8}M and 10^{-7}M)

from stimulating [^{3}H] thymidine uptake into growth arrested rat primary osteoblasts.

Discussion

The MC4-R is likely to play a direct role in bone metabolism since its mRNA is expressed in a rat osteosarcoma cell line as well as in primary rat osteoblasts. The full length mRNA transcript for MC4-R expressed in primary rat osteoblasts is between 2 and 2.6 kb, the correct size for producing a functional protein in these cells. Expression of MC4-R mRNA is, however, much less abundant in osteoblasts than in rat brain, where MC4-R mRNA expression is already considered to be very low compared with many other genes. The MC4-R is not the only melanocortin receptor expressed in osteoblasts since we also observed MC2-R and MC5-R mRNA expressed in very low abundance in primary rat osteoblasts. Despite the low abundance of melanocortin receptors, melanocortin peptides have significant biological effects on osteoblast cell proliferation.

Alpha-MSH ($10^{-9} - 10^{-7}$M) significantly stimulated both thymidine uptake and increased cell number in primary rat osteoblasts. The EC_{50}'s for α-MSH coupling mouse MC4-R and MC5-R to adenylyl cyclase or mobilisation of intracellular calcium are in the 10^{-9}M range, and therefore the α-MSH-stimulated osteoblast proliferation could be mediated by either MC4-R or MC5-R, or both. Alpha-MSH does not stimulate the MC2-R. Surprisingly, $ACTH_{1-24}$ had no significant effect on osteoblast proliferation and yet $ACTH_{1-24}$ functionally couples MC2-R, MC4-R, and MC5-R to adenylyl cyclase when these receptors are overexpressed in various cell lines. Desacetyl-α-MSH (10^{-7}M and 10^{-8}M) also had no significant effect on osteoblast proliferation in two out of three experiments, and yet the EC_{50}'s for desacetyl-α-MSH coupling MC4-R and MC5-R to intracellular signaling pathways when these receptors are overexpressed in heterologous cells are similar to those for α-MSH.

To further understand the significance of MC4-R mRNA expression in osteoblasts we attempted to antagonise the α-MSH stimulated osteoblast

proliferation. Agouti protein is an antagonist of melanocortin peptides coupling MC1-R, MC2-R, and MC4-R. However, in our study agouti protein alone (10^{-9}M– 10^{-7}M) significantly stimulated thymidine incorporation in primary rat osteoblasts and did not antagonise α-MSH stimulated osteoblast proliferation. Furthermore, agouti protein stimulated-thymidine incorporation was not additive with α-MSH stimulated-thymidine incorporation, suggesting that agouti protein and α-MSH may be having their effects through the same melanocortin receptor and signal transduction pathway.

We were unable to distinguish between the three subtypes of melanocortin receptors expressed in osteoblasts based on biological activities of melanocortin receptor agonists, and the MC2-R/MC4-R antagonist, agouti protein. This is not the first time however, that the biological activities of melanocortin receptor ligands on endogenous melanocortin receptors differ from their biological potencies on cloned melanocortin receptors overexpressed in heterologous cells. First, α-MSH and desacetyl-α-MSH are potent agonists of the cloned MC1-R overexpressed in heterologous cell lines, but only α-MSH potently stimulates pigmentation in rodent skin. Second, NDP-MSH is a potent agonist of cloned MC5-R overexpressed in heterologous cell lines, but it is a potent antagonist of α-MSH activation of adenylyl cyclase in 3T3-L1 adipocytes. It is possible that the very low expression of endogenous melanocortin receptors in primary osteoblasts, melanocytes, and 3T3-L1 adipocytes contributes to the differences in melanocortin potencies in these cells compared with overexpressed cloned melanocortin receptors. Additionally, 3T3-L1 adipocytes, like primary osteoblasts, express more than one melanocortin receptor subtype. It is likely therefore, that heterodimeric receptors are formed and these could have different pharmacological profiles from homodimers formed when each cloned melanocortin receptor is overexpressed alone.

Without wishing to be bound by any particular mechanism of action it is proposed that osteoblasts are a model system for understanding

interactions between melanocortin receptor ligands and melanocortin receptors, and this model system more closely resembles *in vivo* responses to melanocortin peptides compared with overexpressing only one melanocortin receptor in an heterologous cell. It has been shown that while desacetyl-α-MSH or $ACTH_{1-24}$ alone had no agonist effects on osteoblast proliferation, they were both capable of antagonising α-MSH stimulated osteoblast proliferation. Desacetyl-α-MSH antagonises α-MSH stimulated mammotrope recruiting activity in anterior pituitary cell cultures and antagonises α-MSH activity on *Anolis* melanophore. Our study is the first time that $ACTH_{1-24}$ antagonism of α-MSH has been reported.

Low level endogenous expression of three melanocortin receptor subtypes in osteoblast cells provides a model system (Figure 8) for exploring interactions between melanocortin receptor ligands and melanocortin receptors that will more accurately reflect the *in vivo* actions of melanocortin peptides, agouti, and agouti gene related peptide. In osteoblasts, and probably many cell types expressing low levels of endogenous melanocortin receptors, there is the likelihood of melanocortin receptor homo- and heterodimers, and cross talk between different melanocortin receptors. These interactions would provide diversity and specificity for melanocortin peptide signalling that would not be available when a single melanocortin receptor subtype is overexpressed in heterologous cells.

It is evident that a variety of cell types and tissues may express melanocortin receptors. In addition to those described above, any such cells or tissues would be appropriate candidates as a biological response system, according to the invention described herein. Examples of cell lines that could be utilised in a similar manner as described above include the GT1-7 mouse hypothalamic cell line, 3T3-L1 adipocytes, melanocytes, L6 myocytes, B16 melanoma cells, and anterior pituitary cell cultures.

Genetically engineered, or heterologous cell lines that stably express a single or a combination of melanocortin peptides are also good

candidates as *in vitro* cellular biological response systems. A panel of such cell lines, each expressing a different melanocortin receptor may comprise a biological response system. Alternatively, co-cultures of two or more heterologous cell lines, each expressing different melanocortin receptors may comprise a biological response system.

Example 5: Biological response by UMR106.06 rat osteosarcoma cell line.

Incorporation of Tritiated Thymidine into DNA

UMR106 cells are plated at at 1 x 10^5 cells/well in a 24 well plate using 10% FCS, DMEM media. 24 hours later the medium is changed to serum free medium containing 0.1% BSA. Following a 24 hour incubation period, the medium is changed again to serum free media containing 0.1% BSA and increasing concentrations of melanocortin peptides. The cells are then incubated for 22 hours. Following this period of incubation {methyl-3H} thymidine [0.5µCi in 25µl/well] is added and left for 2 hours at 37°C (use 0.5µl of 1µCi/µl tritiated thymidine into 24.5µl 0.1% BSA, DMEM for each well). The experiment is terminated by washing the cells with 1ml cold PBS and then add 1ml cold 5% TCA.

The plates are then left at 4°C (on ice) for 15 minutes and then washed 3 x with 1ml cold 5% TCA and twice with 1ml absolute ethanol. The monolayers are air dried and cells dissolved in 1ml 0.3N NaOH by heating at 37°C for 1 hour. 200µl of 1.5N HCL is then added to each well and then the contents of each well is transfered to individual 20ml glass scintillation vials. 7 mls of scintillation fluid is added and mixed well. The samples are counted for 5 minutes.

RESULTS

Figures 9 and 10 show the proliferation response resulting from the treatment of UMR106.06 rat osteosarcoma cells with varying concentrations of alpha-MSH or desacetyl-alpha-MSH.

This example is illustrative of the usefulness of a permanent cell line that can be used as an *in vitro* biological response system. Of course, it will be understood that a proliferative response is only one of many response parameters that may be utilized as a response profile.

Example 6: Use of the *in vitro* biological response system to screen for compounds that act as agonists or antagonists of melanocortin receptors.

An *in vitro* biological response system may be utilised to screen for compounds that act as agonists or antagonists of melanocortin receptors. Such a biological response system could also be utilised to screen for compounds that are useful in the treatment of subjects suffering from obesity and/or type II diabetes, or an imbalance in energy homeostasis or disturbance in feeding/weight gain patterns.

The screening process involves treating the cells of the biological response system having the appropriate combination of receptors with test compounds and then measuring the response parameters, either by mass spectrometry or by gene expression array or by other available techniques which are able to assess the required response parameters. The compound that produces the desired response profile is a compound which may be useful in the treatment of obesity and/or type II diabetes or imbalances in energy homeostasis and/or disturbances in feeding/weight gain patterns. The biological response system will also enable the selection of compounds that are able to block the undesirable effects of environmental and nutritional factors that cause obesity and/or type II

diabetes or imbalances in energy homeostasis and/or disturbances in feeding/weight gain patterns.

The profile generated by compounds that produce a desired response in an *in vitro* biological response system may then be compared with the profile that is generated from the administration of the compound to an *in vivo* biological response system.

Although the invention has been described with reference to specific examples, it will be appreciated by those skilled in the art that the invention may be embodied in many other forms.

DATED this 23rd day of August, 2002

Auckland Uni. Services.

Attorney: Ivan Rajkovic
Fellow Institute of Patent and Trade Mark Attorneys of Australia
of BALDWIN SHELSTON WATERS

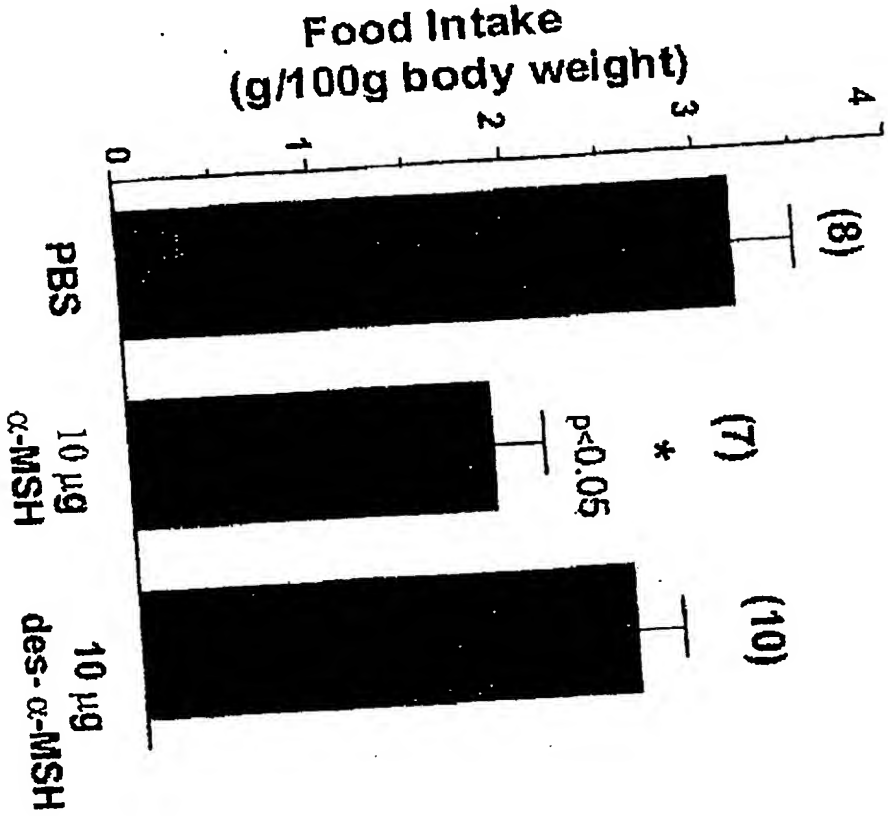

Food Intake
(g/100g body weight)
0
1
2
3
4
PBS
(8)
HSW-α-MSH 10 µg
(7)
*
P<0.05
HSW-α-des-MSH 10 µg
(10)

FIG 1.

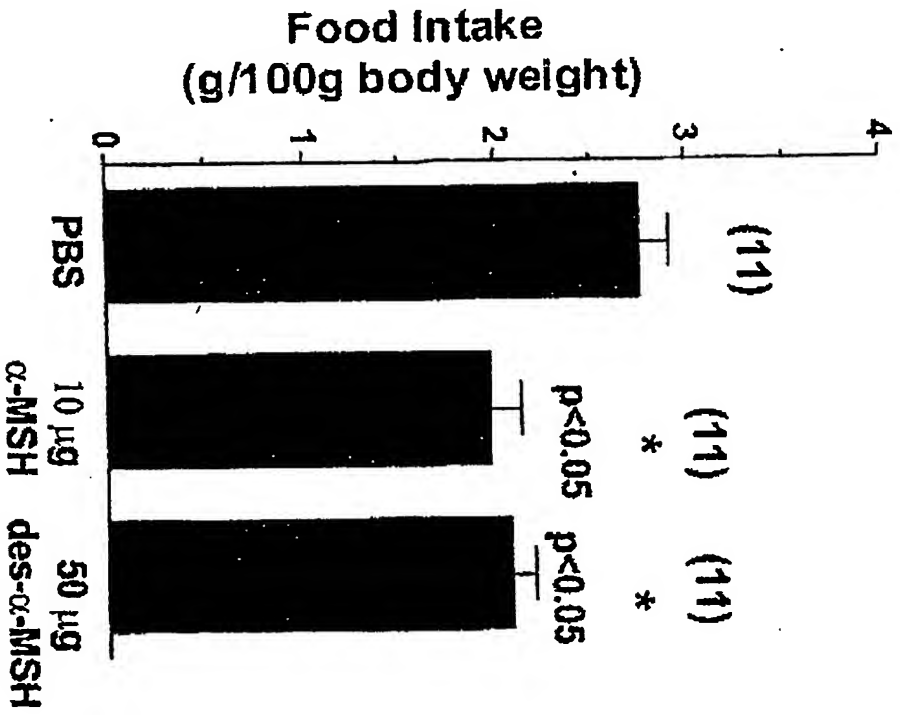

Food Intake
(g/100g body weight)
0
1
2
3
4
PBS
(11)
α-MSH 10 µg
p<0.05
*
(11)
des-α-MSH 50 µg
p<0.05
*
(11)

FIG-2

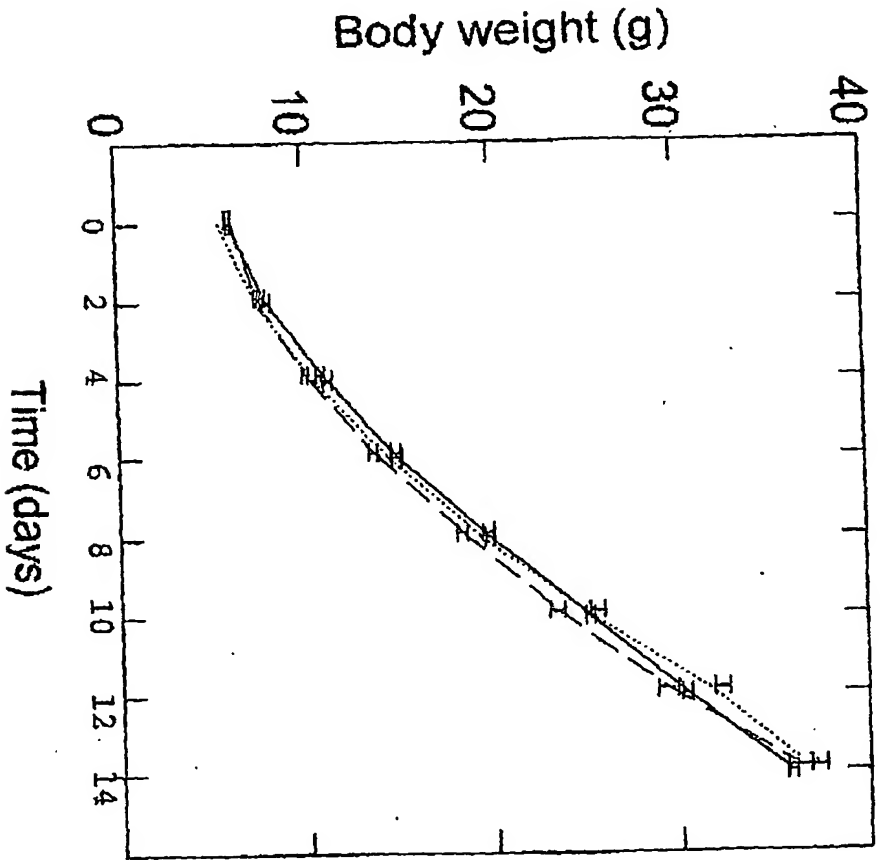


FIG 3.

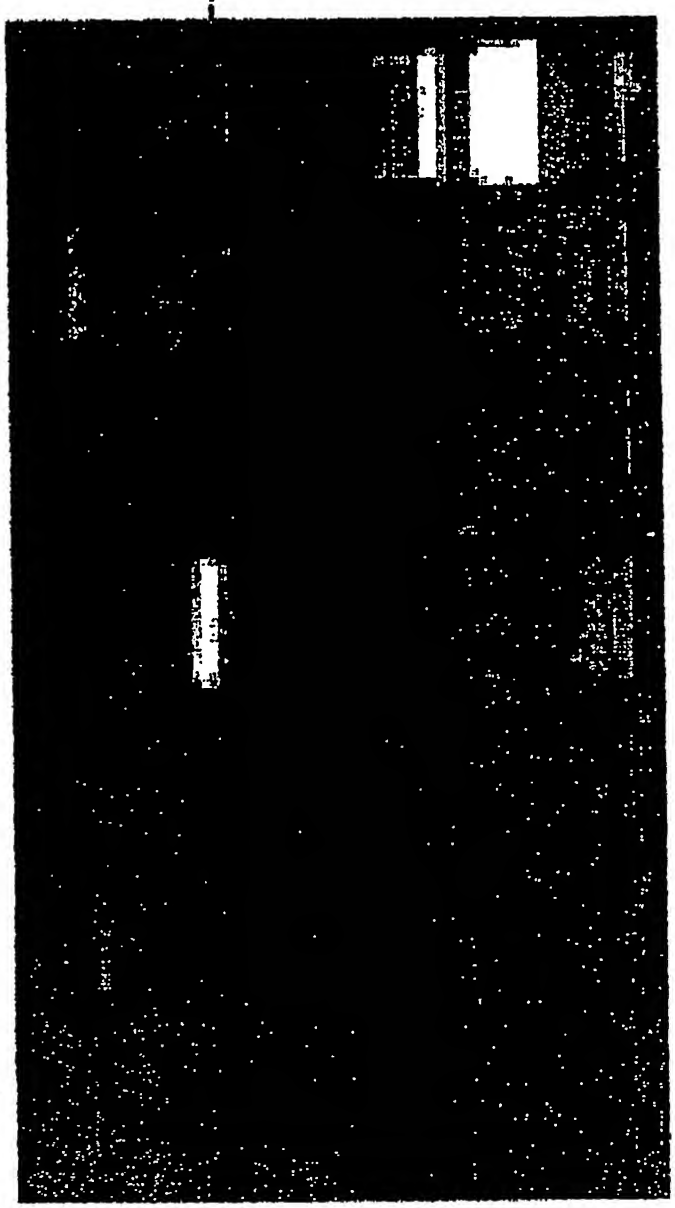


FIG 4.

FIG 5.

FIG 6.

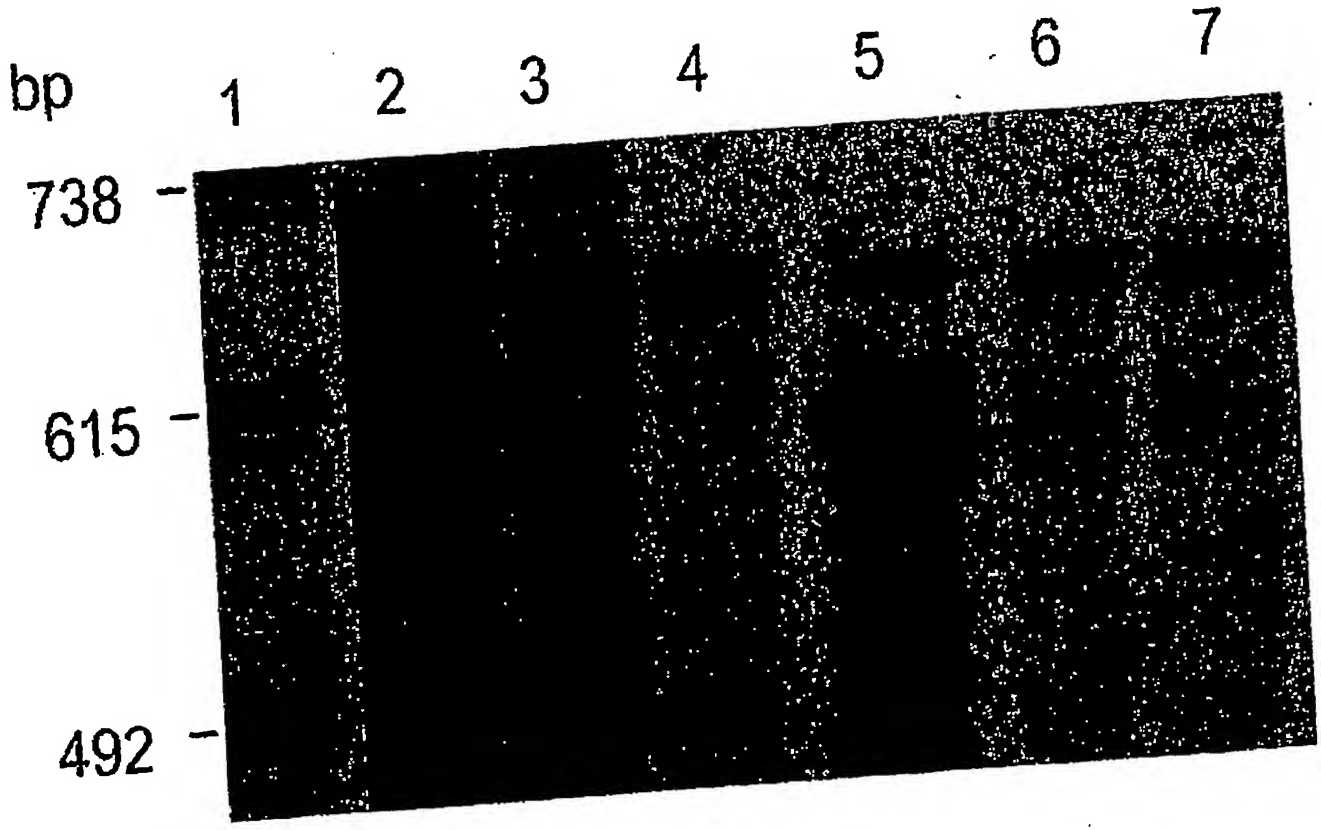
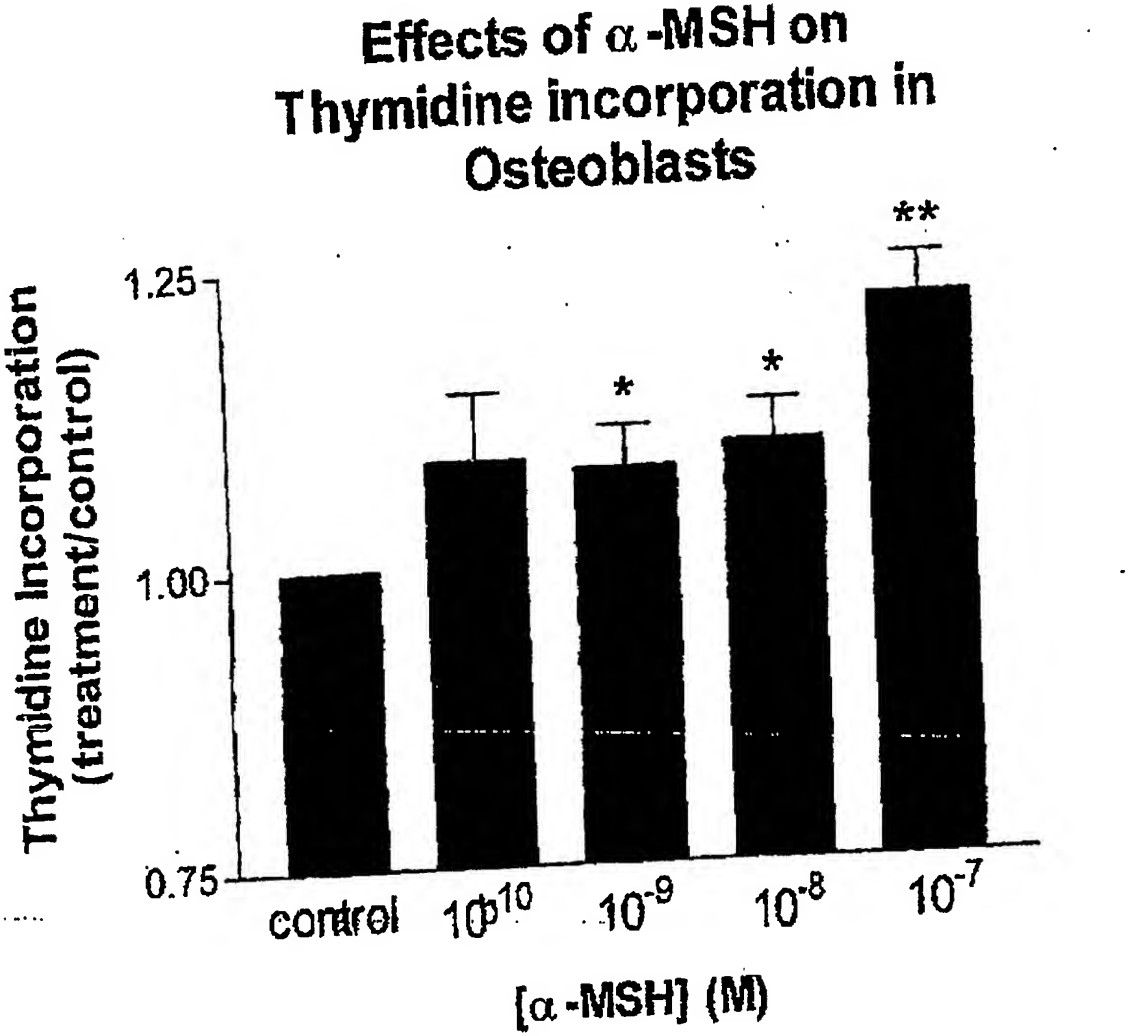


FIG 7.



*=significantly different from control($p<0.04$)

**=significantly different from control ($p<0.001$)

64 9 3737499

Des-acetyl α-MSH is Antagonistic to α-MSH in Osteoblasts

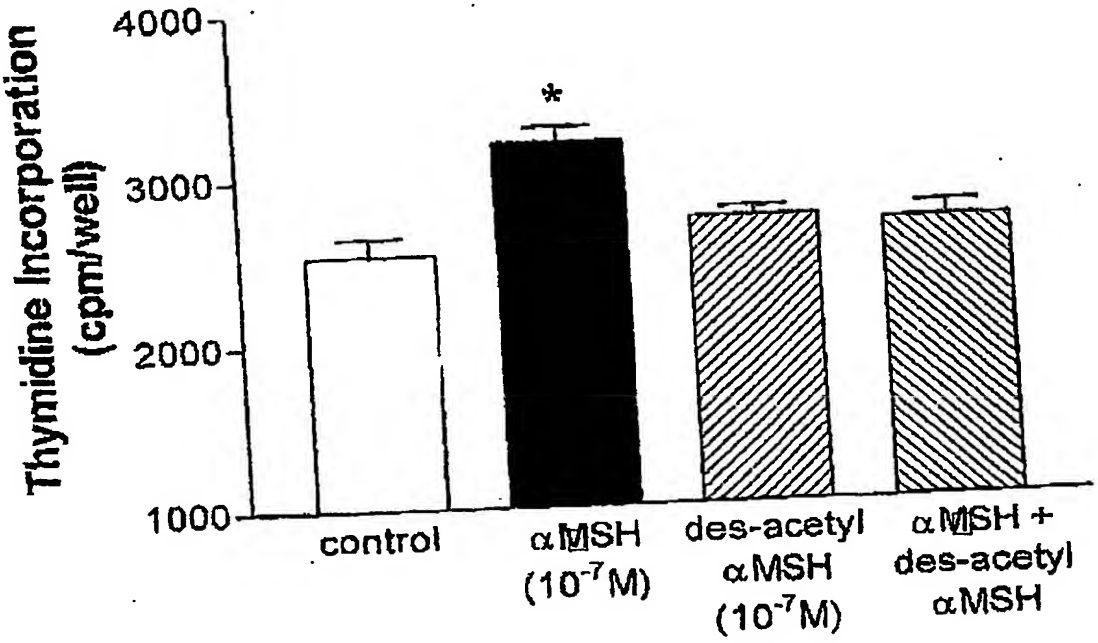


Fig 8

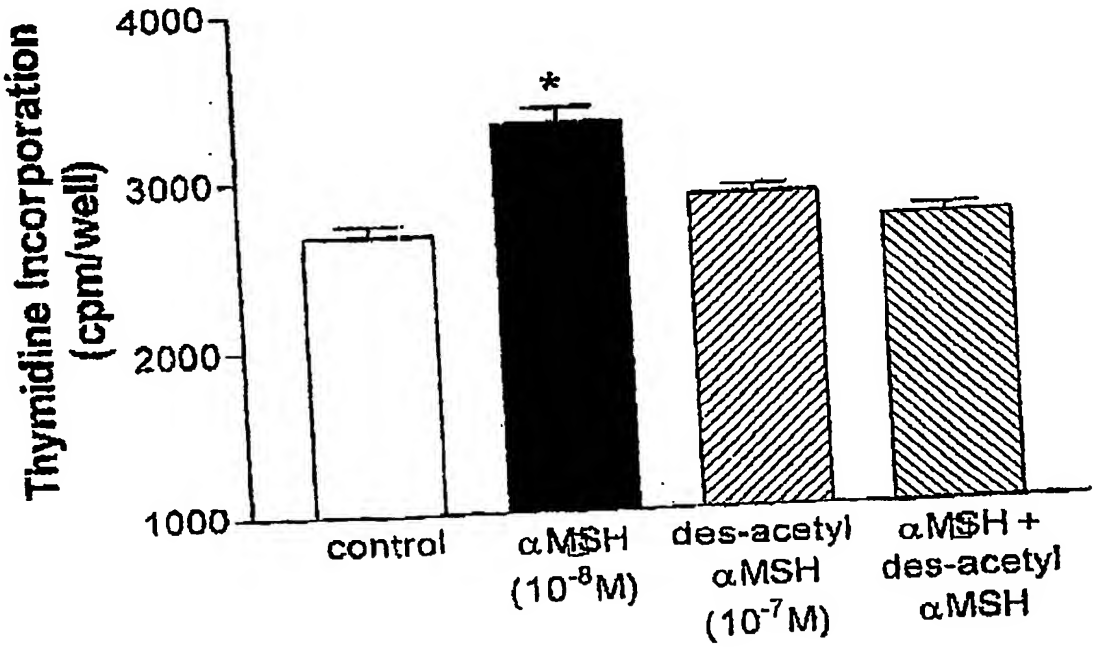


FIG. 9

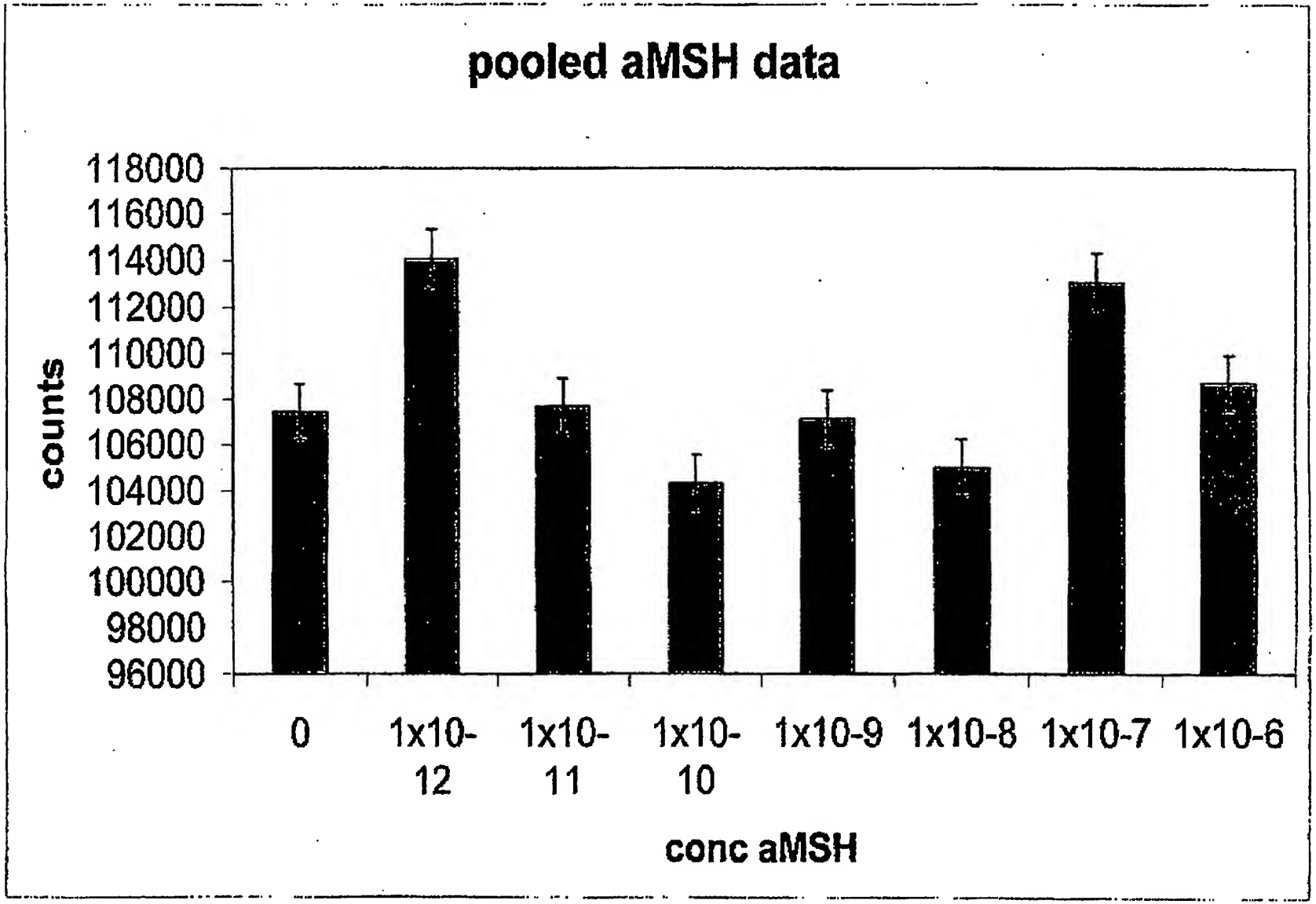


FIG. 10.

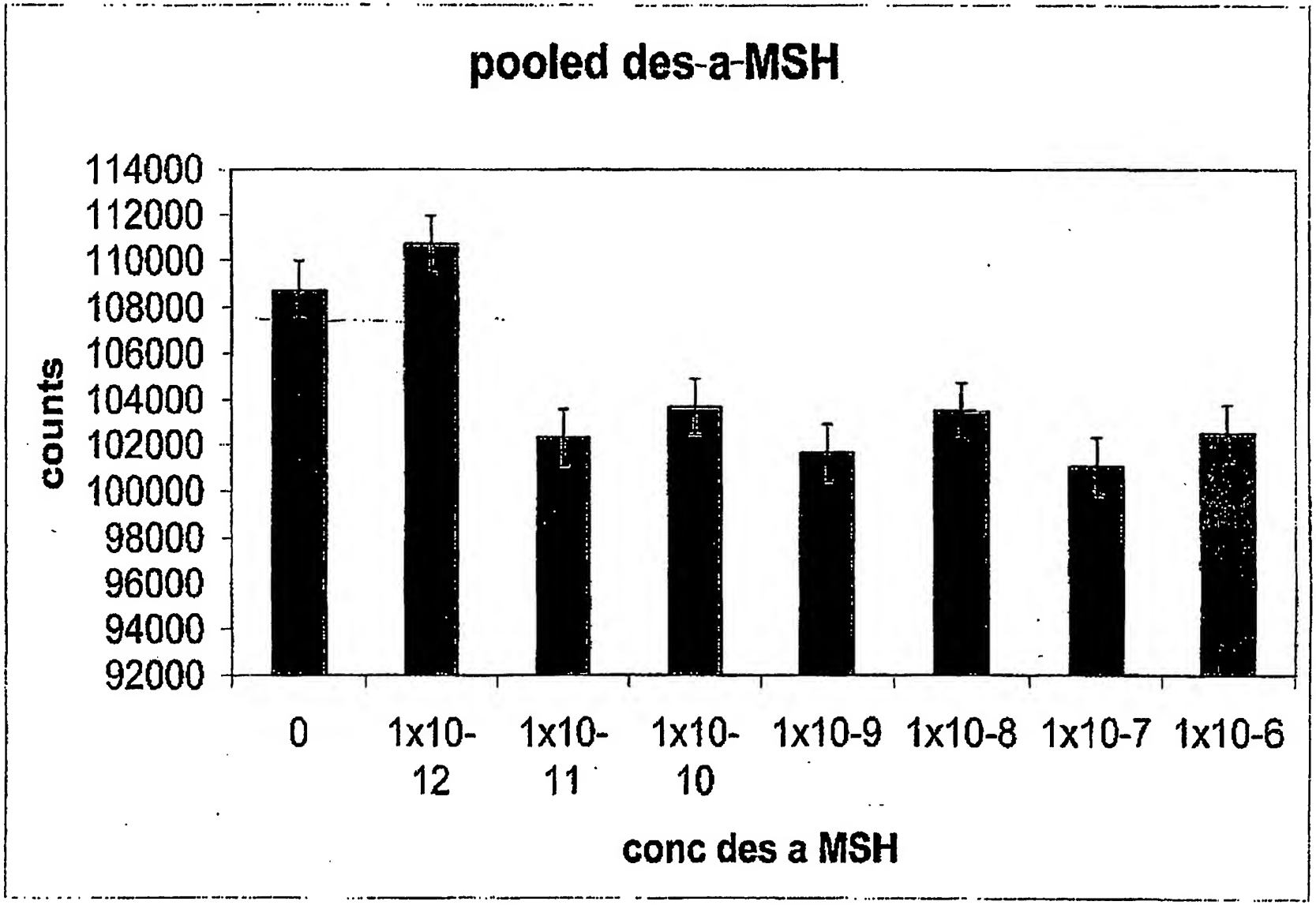


FIGURE IIA

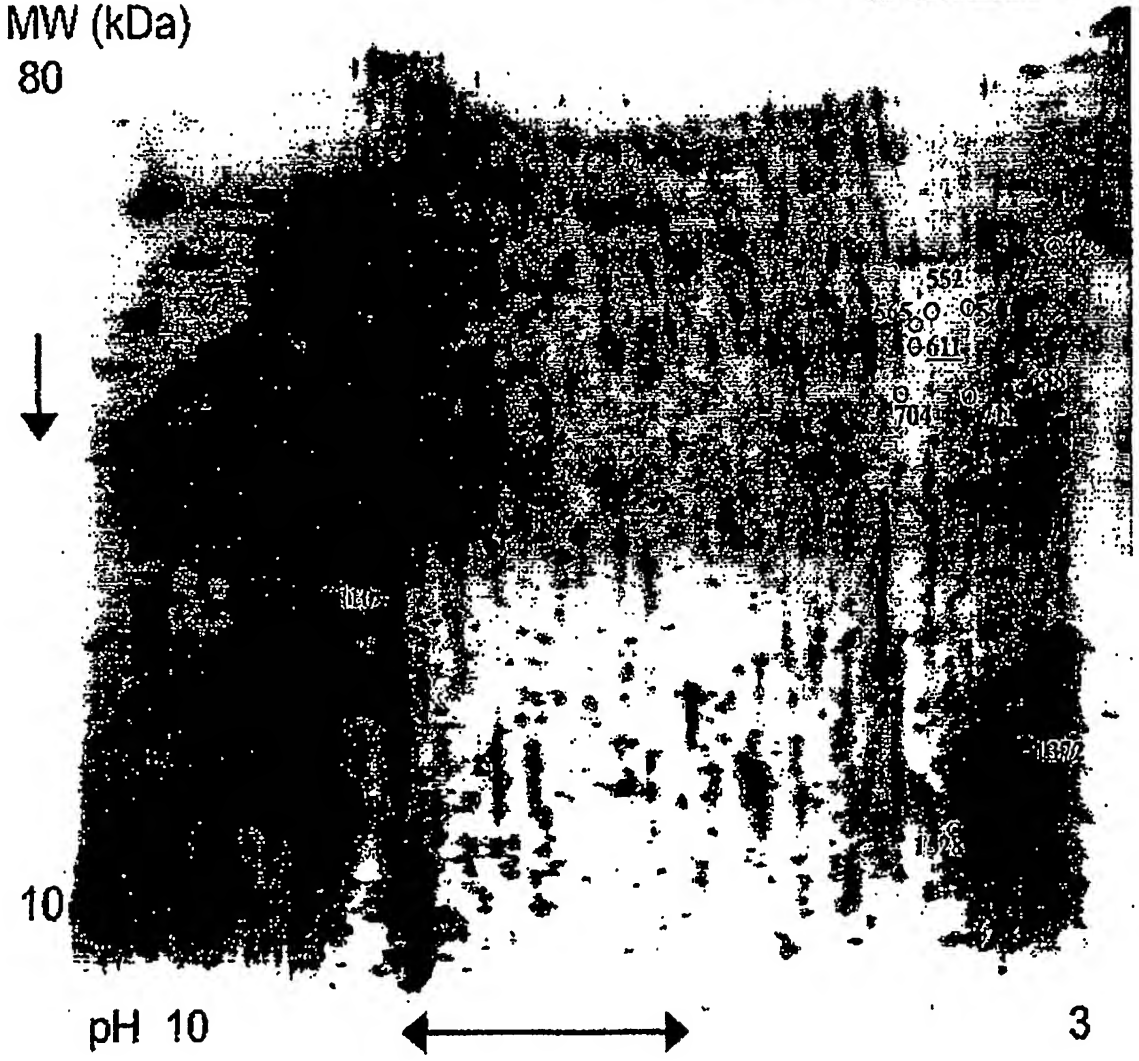
Examples of proteins identified

Protein	Spot no. (Fig 3,4)	α-MSH effect compared to control	des-α-MSH effect compared to control
Stress protein			
heat shock protein homologue	p1350 (3)	1.8 fold increase	
Enzymes			
Glyceraldehyde-3-phosphate-dehydrogenase	p1528 (3)	*	
	p582 (4)		*
	p1210 (4)		2.4 fold decrease
aldo-keto reductase	p582 (4)		*
citrate synthase	p582 (4)		*
creatine kinase	p706 (3)	1.7 fold increase	
pyruvate synthase alpha-chain	p86 (4)		1.6 fold decrease
f1 ATPase beta-chain	p1528 (3)	*	
Cytoskeletal proteins			
tubulin beta chain	p711 (3)	1.4 fold increase	

* Multiple proteins that appeared as a single spot on the 2D gel.

FIGURE 11B

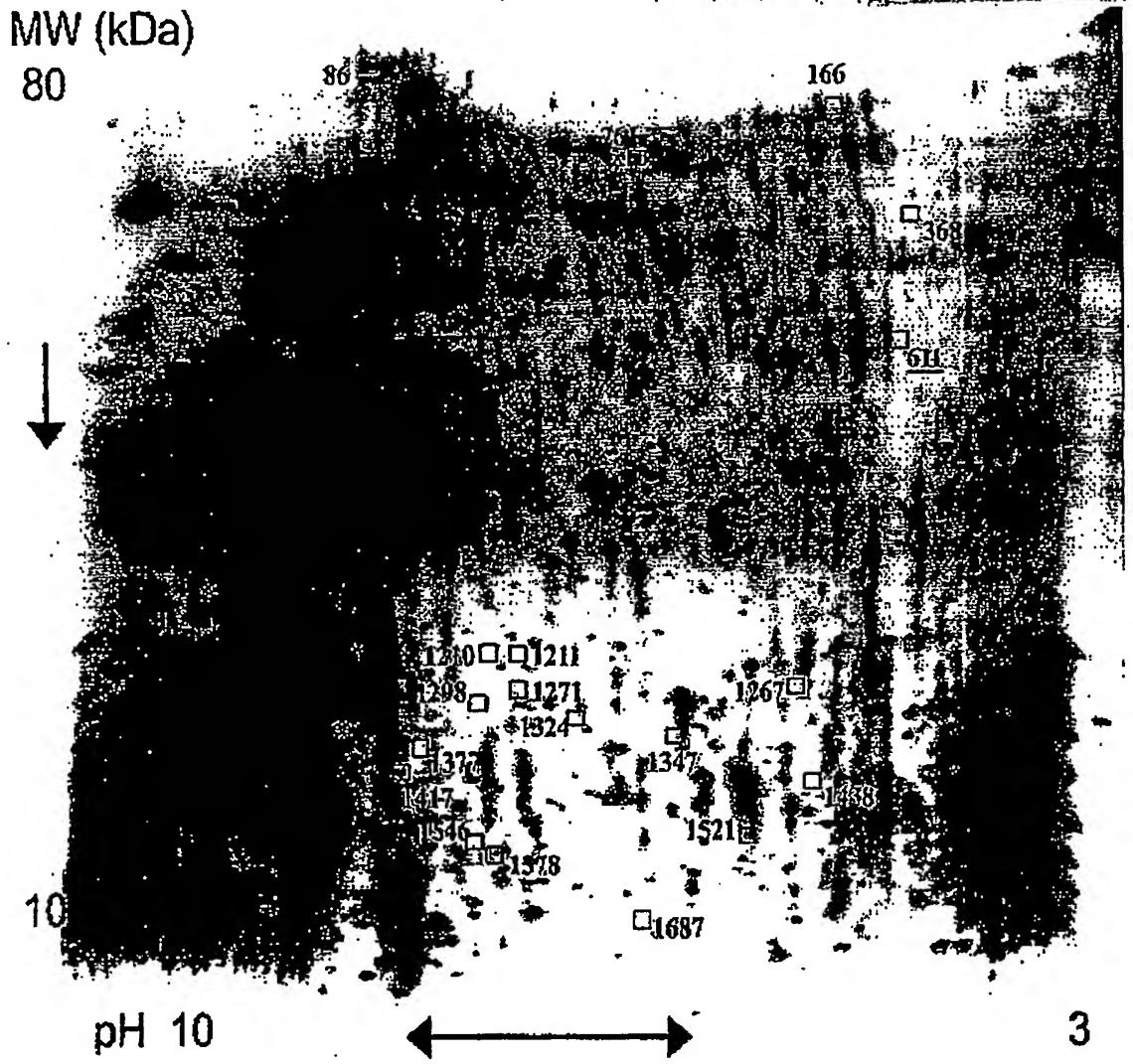
Proteins changed by α-MSH treatment



Location of proteins of interest (black-circles) on the reference gel. Numbers correspond to protein labels on the reference gel. Significant increase ($p<0.05$, Mann-Whitney U test) in protein expression in α-MSH treated group is labeled in blue, decrease in red. Underlined protein p611 expression also significantly changed in des-α-MSH treated group.

FIGURE 11C

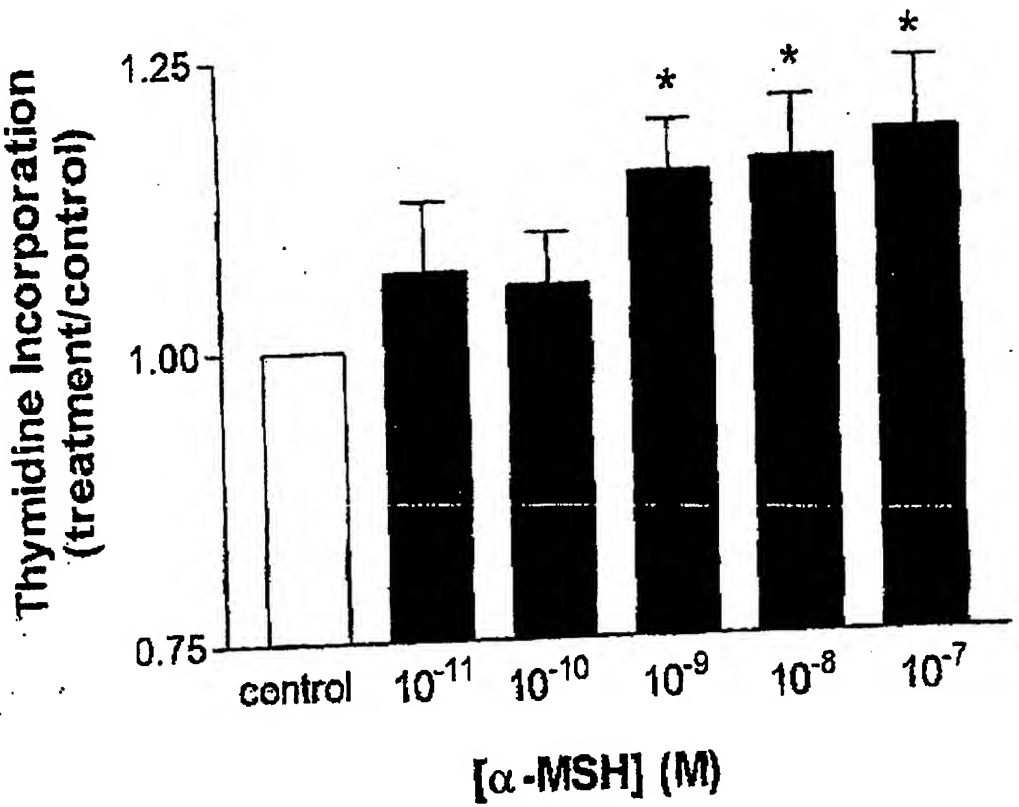
Proteins changed by des-α-MSH treatment



Location of proteins of interest (black-squares) on the reference gel. Numbers correspond to protein labels on the reference gel. Significant increase ($p<0.05$, Mann-Whitney U test) in protein expression in des-α-MSH treated group is labeled in blue, decrease in red. Underlined protein p611 expression also significantly changed in α-MSH treated group.

FIG-12.

Effects of α-MSH on Thymidine Incorporation in Chondrocyte Monolayers



*=significantly different from control ($p<0.03$)